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The Importance of Loop Region Residues 40–46 in Human Dihydrofolate Reductase as Revealed by Site-directed Mutagenesis*

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Site-directed mutagenesis has been used to delete 2 residues (Gly⁴⁵-Lys⁴⁶) from a flexible "loop" region between residues 40 and 46 of human dihydrofolate reductase. Steady-state kinetic studies show that the K_m values for the deletion mutant enzyme for both dihydrofolate and nicotinamide adenine dinucleotide phosphate (reduced) (NADPH) as well as the pH rate profile are virtually identical to that of the wild type. In contrast, the V_{max} value of the mutant enzyme is decreased 2.5-fold. The results suggest that the loop region may play a role in the catalytic efficiency but not necessarily in the binding of substrates. Agents such as KCl, urea, and organomercurials at concentrations which show activating effects on the wild-type human dihydrofolate reductase have little or no effect on the deletion mutant. Competitive enzyme-linked immunosorbent assay experiments using peptide-specific antibodies against cyanogen bromide fragments generated from human dihydrofolate reductase show that the binding of folate, NADPH, and methotrexate, either in binary or in ternary complexes with the wild-type enzyme, causes a striking reduction in the binding of the antibodies. Compared with wild type, the binding of these ligands with the deletion mutant enzyme causes much less inhibition (2–16-fold less) in the binding of all three antibodies. The altered properties of the mutant enzyme can be explained on the basis of a need for the flexible loop 40–46 for reversible protein unfolding during activation and also for conformational changes induced by ligand binding, thus "communicating" the effects of ligand binding.

Conformational changes are frequently invoked to account for the action of enzymes even though there is often little or no information regarding the molecular distortions presumed to occur at the active site and in other regions of the protein molecule. Elucidating the nature of such changes is important. In some cases, it is assumed that the binding of a ligand to the active site results in the enzyme being converted to a more active conformation and/or a conformation with higher affinity for substrate. Dihydrofolate reductase (5,6,7,8-tetrahydrofolate:NADP⁺ oxidoreductase, EC 1.5.1.3) (DHFR),¹ an im-

portant enzyme for maintaining the intracellular pools of tetrahydrofolate and its derivatives, is a valid model for investigating these effects.

A critical factor in the efficiency of interaction of substrate or folate analogs and NADPH with DHFR is the mutual enhancement (up to 1000-fold) of their affinity for the enzyme via structural changes induced in the active site (1–4). The presence of different conformational states of DHFR in its free form *versus* complexes with NADPH or folate analogs has been established by NMR and fluorescence studies (5–9) for bacterial DHFR and by x-ray crystallography for bacterial and vertebrate DHFRs (10–12). Our previous studies (13, 14) showed that human DHFR (hDHFR) upon binding the ligands folate, NADPH, and methotrexate (MTX)-NADPH decreased the binding ability of polyclonal anti-hDHFR peptide antibodies in regions distant to the active site. These results suggested the possible role of regions of the protein distant from the active site in communicating the effects of ligand binding. Since the secondary structural elements, *i.e.* α -helices and β -sheets, were not disrupted by ligand binding as observed by x-ray crystallography our results indicated that the vulnerable antigenic epitopes must reside in the flexible "loop" regions.

Thereafter, in the companion paper (15), we have systematically mapped the antigenic epitopes of hDHFR which confirmed our earlier prediction (14) that most of the epitopes reside in loop regions at the boundaries of secondary structural elements (15). These regions are ideal targets for using protein bioengineering methods to study conformational changes as related to the functions of DHFR. For this purpose, we used oligodeoxynucleotide-directed mutagenesis to delete the two residues (Gly⁴⁵, Lys⁴⁶) from a loop located at residues 40–46 from the hDHFR cDNA. This loop is a high thermally mobile region (16) and is 15–25 Å away from the active site (17). The mutant hDHFR des[Gly⁴⁵-Lys⁴⁶] has been characterized with respect to its steady-state kinetics, activation properties, and protein structure alterations upon ligand binding using antibody probes. Our results provide information about the important role of this flexible loop region in the function of hDHFR.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases, T4 polynucleotide kinase, and T4 DNA ligase were purchased from Bethesda Research Laboratories, U. S. Biochemicals, and Boehringer Mannheim, respectively. The oligonucleotide-directed *in vitro* mutagenesis system version 2 and [α -³⁵S]dATP were purchased from Amersham Corp. DNA sequencing was carried out using the sequence kit from U. S. Biochemicals. MTX was a generous gift from Dr. John A. R. Mead (Division of Cancer Treatment, National Cancer Institute). Dihydrofolate (FAH₂) was prepared from commercial folic acid by dithionite reduction (18).

Construction of the Human DHFR (DNA) Deletion Mutant—A 20-base oligodeoxynucleotide probe (CTTCAGTAGAAGGT AAA CAG

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¹ The abbreviations used are: DHFR, dihydrofolate reductase; hDHFR, human dihydrofolate reductase; MTX, methotrexate (4-amino-10-methyl-4-deoxyfolate); FAH₂, dihydrofolate; SDS, sodium dodecyl sulfate; pHMB, *p*-(hydroxymercuri)benzoate; MES, 2-(*N*-morpholine)ethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; dCTP α S, deoxycytidine triphosphate where a sulfur atom is substituted for an oxygen atom in the α position.

AATCTG) used for deletion mutagenesis was synthesized using an Applied Biosystems model 380A automated DNA synthesizer and was purified on 15% polyacrylamide gel as described by Applied Biosystems (DNA Synthesizer User Bulletin 13, 1984). The underlined base triplets coding for Gly⁴⁵ and Lys⁴⁶, respectively, were deleted. The single-stranded DNA template for the mutagenesis reaction was prepared by cloning part of wild-type hDHFR cDNA from plasmid pDFR, an expression vector for the hDHFR cDNA (19), into M13. Deletion mutagenesis was carried out using an oligonucleotide-directed *in vitro* mutagenesis system. Phosphorylation of the mutagenic oligonucleotide, annealing of the oligonucleotide with DNA template, extension of the oligonucleotide with the Klenow fragment of *Escherichia coli* DNA polymerase I with dCTPaS in place of dCTP, filtering of the reaction mixture to remove single-stranded DNA, nicking the wild-type strand with *NciI*, removal of the nicked strand with exonuclease III, and repolymerization with *E. coli* DNA polymerase I were carried out according to the instructions of the manufacturer. The final product was used to transform *E. coli* TG1. Single-stranded DNA was prepared from five plaques, and DNA from each plaque was sequenced (single lane) using the dideoxy sequencing method (20, 21) to detect the mutation.

For cloning of the mutant hDHFR cDNA back into the pDFR expression vector, the replicative form of M13-containing mutant cDNA was prepared and digested with *EcoRI* and *PstI* to obtain the mutant cDNA fragment. pDFR DNA was digested with *EcoRI* and *PstI*, and the pDFR vector fragment was then ligated with the mutant hDHFR cDNA fragment. The ligation mixture was used to transform *E. coli* JM107.

The plasmid carrying the mutant hDHFR gene was sequenced by the double-stranded dideoxy method (20, 22). To facilitate this work, three synthetic primers were used dividing the fragment to be sequenced into three parts of about 200 base pairs each in order to cover the entire 558-nucleotide coding sequence.

Expression and Purification of Mutant hDHFR des[Gly⁴⁵-Lys⁴⁶].—JM107 cells containing the mutant plasmids were grown in a 14-liter culture at 37 °C in the presence of 50 mg of ampicillin/ml to an optical density of 0.6–0.7 (at 600 nm) and induced by the addition of 5 g of isopropyl- β -D-thiogalactopyranoside. After further growth for 3–4 h, the cells were harvested by continuous flow centrifugation at 15,000 rpm at 4 °C. The cell pellet was stored at –70 °C. The following steps were carried out at 4 °C. Cells from the 14-liter culture were resuspended in 300 ml of buffer A (50 mM potassium phosphate, pH 7.5, 1 mM EDTA, 10 mM β -mercaptoethanol with 1 mM phenylmethanesulfonyl fluoride, and 2 mg/liter leupeptin (lysis buffer). Lysozyme (150 mg/7 ml of lysis buffer) was added to the suspension followed by incubation on ice for 15 min. $MgCl_2$ (1 M, 900 μ l) and 24 mg of DNase I were added, and the suspension was placed on ice for another 15 min. The suspension was then centrifuged at 12,000 rpm for 15 min. To the supernatant, ammonium sulfate was slowly added to a final concentration of 85% with gentle stirring overnight. Following centrifugation at 18,000 rpm for 30 min, the pellet was resuspended in 250 ml of lysis buffer and loaded onto a 1.5 \times 3-cm MTX-aminohexyl-Sepharose 4B column equilibrated with buffer A. After extensive washing with buffer B (buffer A + 500 mM KCl) and buffer C (buffer B, pH 8.0) the column was eluted with FAH₂ (2 mg/ml in buffer C). The fractions containing DHFR activity were pooled and concentrated to 10 ml in an Amicon ultrafiltration apparatus fitted with a YM-10 membrane and stored at 4 °C. The enzyme was then passed through a 1 \times 30-cm Sephadex G-50 column equilibrated with buffer A to remove excess substrate. Enzyme purity was evaluated by SDS-polyacrylamide gel electrophoresis (23).

Protein Determination.—Enzyme concentration was determined by both MTX titration (24) and by the Bio-Rad version of Bradford's dye-binding assay (25). MTX concentration was determined spectrophotometrically at 258 nm ($E_m = 23,250$) and 302 nm ($E_m = 22,100$) (26).

Steady-state Kinetics.—DHFR activity was determined by measuring the decrease in absorbance at 340 nm and at 22 °C which accompanied the conversion of FAH₂ to tetrahydrofolate and of NADPH to NADP⁺ (27, 28). Initial rates were derived from the change in absorbance continuously recorded with a Varian model 219 spectrophotometer in a reaction buffer containing 50 mM Tris-HCl, pH 7.5. FAH₂ concentration was determined both spectrophotometrically at 282 nm (extinction coefficient = 2.8×10^4 M^{–1} cm^{–1}) and 340 nm (extinction coefficient = 6.59×10^3 M^{–1} cm^{–1}) (18). NADPH concentration was determined in the same way using a molar extinction coefficient of 6.2×10^3 M^{–1} cm^{–1} at 340 nm and 1.49×10^4 M^{–1} cm^{–1} at 259 nm (29). The K_m values for FAH₂ and NADPH were determined by varying the concentration of one substrate while

maintaining the concentration of the second substrate at a constant saturating level and by measuring the initial velocity at each concentration. The data were fit to the following Michaelis-Menten equation.

$$V = \frac{V_{\max} [S]}{K_m + [S]}$$

Use of a nonlinear regression program yielded values for V_{\max} and K_m . The k_{cat} was calculated by dividing V_{\max} by the concentration of enzyme used in the reaction. To maintain constant ionic strength over the pH range used in obtaining pH profiles of k_{cat} , a three-component buffer system was used containing 50 mM Tris, 25 mM acetate, 25 mM MES, and 100 mM NaCl (MATS buffer).

Anti-peptide Antibodies and ELISA.—The production of antibodies against CNBr peptides 15–52, 53–111, and 140–186 derived from hDHFR has been described (14). Denatured hDHFR, obtained by precipitation with 10% trichloroacetic acid followed by boiling in 1% SDS and diluting to <0.05% SDS, was immobilized in a microtiter plate (0.1–0.2 μ g/well). Antisera preincubated in the absence and in the presence of native and various ligand-complexed forms of DHFR (see the legend for Fig. 6) in 10 mM sodium phosphate, pH 7.5, containing 150 mM sodium chloride, 0.1% Tween 20, and 1% nonfat dry milk were assayed in these wells using glucose oxidase-conjugated goat-anti-rat IgG and measuring the absorbance at 405 nm in the presence of horseradish peroxidase, β -D-glucose, and 2,2'-azino-bis(3-ethylbenzothiazoline 6-sulfonic acid) as described (30).

RESULTS

Construction of the Mutant.—The deletion mutant hDHFR des[Gly⁴⁵-Lys⁴⁶] was constructed by oligodeoxynucleotide-directed mutagenesis, which is based on the method of Eckstein and co-workers (31, 32), with mutagenesis efficiencies of >80%. After the mutant hDHFR cDNA was cloned into the pDFR vector, the entire structural gene was sequenced demonstrating that the expected deletion had been introduced and that no other alteration had occurred (data not shown).

Stability of the Mutant Enzyme.—The thermal stability experiments (Fig. 1) showed that the mutant des[Gly⁴⁵-Lys⁴⁶], in its apoenzyme form, lost more than 80% activity in less than 30 min at 22 °C unlike the wild-type DHFR which remained fully active. The NADPH-enzyme and FAH₂-enzyme complexes, like their counterparts in the wild-type enzyme, are quite stable (Fig. 1). The FAH₂-enzyme complex could be stored at 4 °C up to 3 weeks with greater than 90% activity remaining (data not shown).

Kinetic Studies.—The steady-state kinetic properties of the wild-type and mutant hDHFRs were examined in order to make a preliminary assessment of the effects of the mutation on the substrate binding properties of the enzyme. Specific activities were determined by MTX titration based on the fact that MTX is a stoichiometric tight binding inhibitor of DHFR (24). The titration curves for both wild-type and des[Gly⁴⁵-Lys⁴⁶] enzymes are shown in Fig. 2. There is a linear relationship between MTX concentration and inhibition of

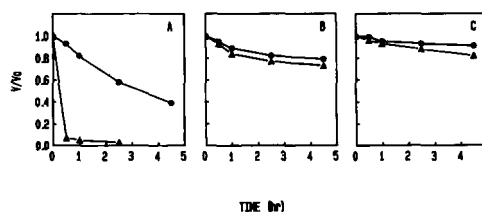


FIG. 1. Thermal stability of wild type (●) and des[Gly⁴⁵-Lys⁴⁶] (▲) hDHFRs at 22 °C. The enzymes (0.1 nM) were incubated in 50 mM Tris-HCl, pH 7.5. Aliquots were removed from the incubation mixture at various time intervals as indicated and assayed. V_0 , DHFR activity at time 0; V , DHFR activity at the indicated time. A, enzyme alone; B, enzyme + 0.1 mM NADPH; C, enzyme + 0.1 mM FAH₂.

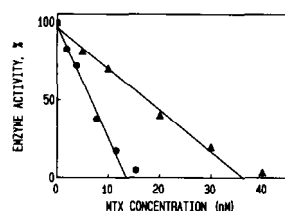


FIG. 2. Methotrexate titration of wild-type (●) and des[Gly⁴⁵-Lys⁴⁶] (▲) DHFRs. The enzymes with the same units of activity were incubated with various concentrations of MTX in 50 mM Tris-HCl, 100 μ M NADPH, pH 7.5, for 2 min at 22 °C. The DHFR activities were monitored after addition of 50 μ M FAH₂ for at least 1 min.

TABLE I

Steady-state properties of wild-type and mutant des[Gly⁴⁵-Lys⁴⁶] DHFRs

Steady-state properties were determined by spectrophotometric measurements of initial velocities as a function of substrate concentration in 50 mM Tris-HCl buffer, pH 7.5. All reactions were measured at 22 °C.

Enzyme	Michaelis constants		k_{cat}	Specific activity
	FAH ₂	NADPH		
	μ M		s^{-1}	units/mg
Wild type	0.032	0.26	7.3	20
des[Gly ⁴⁵ -Lys ⁴⁶]	0.036	0.29	2.9	7.8

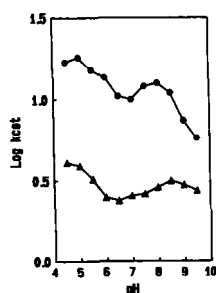


FIG. 3. pH dependence of k_{cat} for the wild-type (●) and deletion mutant (▲) DHFRs. Reaction conditions were 22 μ M NADPH, 10 μ M FAH₂, 0.47 nM des[Gly⁴⁵-Lys⁴⁶], and 0.34 nM wild-type hDHFR at 22 °C in MATS buffer.

enzyme activity up to a 90% loss of activity for both wild type and mutant. The specific activity of the des[Gly⁴⁵-Lys⁴⁶] enzyme is 7.8 units/mg (corresponding to a k_{cat} of 2.8 s^{-1}), whereas the specific activity of wild type is 20 units/mg (corresponding to a k_{cat} value of 7.3 s^{-1}) (19). Thus, the k_{cat} for the wild-type enzyme is about 2.5-fold greater than that for des[Gly⁴⁵-Lys⁴⁶].

The K_m values for FAH₂ and NADPH for the des[Gly⁴⁵-Lys⁴⁶] mutant reductase were determined and the results compared to those of wild-type enzyme (Table I). Apparent K_m values for FAH₂ and for NADPH for the mutant are approximately the same as those found for the wild-type enzyme (33). The pH dependence of k_{cat} for both mutant and wild-type hDHFR were determined, and the pH rate profiles are shown in Fig. 3. The des[Gly⁴⁵-Lys⁴⁶] hDHFR showed two pH optima, one at about pH 4–4.5 and another at about pH 8–8.5, similar to that for the wild type (34, 35).

Effects of Activating Agents—Human DHFR can be activated to different extents by KCl, urea, and organic mercurial compounds such as *p*-hydroxymercuribenzoate (pHMB) (34). The effects of these activating agents were tested on the mutant enzyme. Fig. 4 shows the effects of KCl and urea on the enzymic activities of the wild-type and mutant reductases.

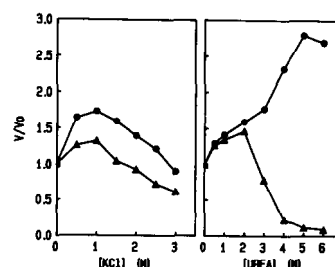


FIG. 4. The effects of urea and KCl on the enzymic activity of wild-type (●) and des[Gly⁴⁵-Lys⁴⁶] (▲) DHFRs. The enzymes were assayed in the presence of various concentrations of urea or KCl as indicated in 50 mM Tris-HCl, pH 7.5, at 22 °C. V_0 , DHFR activity in the absence of urea or KCl; V , DHFR activity at the indicated concentration of urea or KCl.

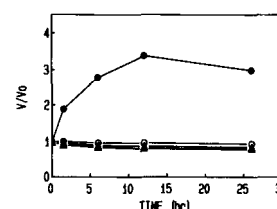


FIG. 5. The effect of pHMB on wild-type (●) and des[Gly⁴⁵-Lys⁴⁶] (▲) DHFRs. The enzymes (0.8–1 μ M) were incubated with a 100-fold molar excess of NADPH in 50 mM potassium phosphate, pH 7.5, at 22 °C in the presence of a 10-fold molar excess of pHMB (●, ▲) and in the absence of pHMB (○, △). Aliquots were removed from the incubation mixture and assayed at the times indicated. V_0 , DHFR activity in the absence of pHMB at time 0; V , DHFR activity at the indicated time.

Wild-type enzyme was activated to about 1.8-fold at 1 M KCl, whereas the mutant was activated significantly less (about 1.3-fold) under the same conditions. The wild-type enzyme was activated about 2.8-fold at a 5 M concentration of urea whereas des[Gly⁴⁵-Lys⁴⁶] hDHFR showed a slight activation at low concentrations with about a 1.4-fold activation at 2 M urea and a rapid decline in activity at concentrations higher than 2 M. Fig. 5 illustrates the effects of pHMB on the enzyme activities of wild-type and the deletion mutant. As shown in Fig. 5, wild-type enzyme was activated about 4-fold when incubated with a 10-fold molar excess of pHMB in the presence of NADPH, whereas des[Gly⁴⁵-Lys⁴⁶] was not activated under the same conditions.

Binding of Antibodies to Wild Type, des[Gly⁴⁵-Lys⁴⁶], and Their Enzyme-Ligand Complexes—The binding of three peptide-specific antibodies to DHFR in solution was determined by competitive ELISA assays in which denatured hDHFR was immobilized. The binding of antibodies, at an appropriate dilution, to immobilized antigen was assayed in the presence of various concentrations of native wild-type or des[Gly⁴⁵-Lys⁴⁶] hDHFR or in the presence of the enzyme preincubated with a saturating concentration of folate (0.1 mM), NADPH (0.1 mM), or MTX (0.2 mM) plus NADPH. As indicated in Fig. 6, in the case of all three antisera, native wild-type or deletion mutant hDHFR competed for the antibodies, inhibiting their binding to the immobilized denatured enzyme in a concentration-dependent manner. Preincubation of the wild-type hDHFR with folate, NADPH, or MTX plus NADPH significantly inhibited antibody binding to the native enzyme to various degrees, shifting the curve to the right in all cases (Fig. 6A, I–III). Maximum inhibition of antibody binding to the native enzyme was produced when MTX and NADPH were both bound in a ternary complex. In the case of des[Gly⁴⁵-Lys⁴⁶], however, the preincubation of folate, NADPH, or MTX plus NADPH compared with native wild

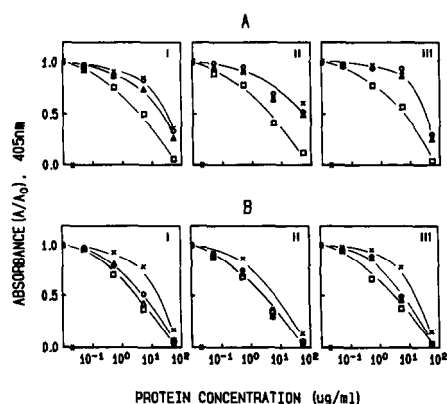


FIG. 6. Competitive immunoassays using antisera to peptides 15–52 (I), 53–111 (II), and 140–186 (III). ELISA assays were carried out with denatured hDHFR as the immobilized antigen. Antisera to cyanogen bromide peptides 15–52, 53–111, and 140–186 were used at 1/5000, 1/1000, and 1/4000 dilutions, respectively, due to differences in antibody titers. The antisera (50 μ l) were preincubated for 2 h at 22 $^{\circ}$ C in the absence or in the presence of various concentrations of: A, wild-type hDHFR or its complexes with ligands; B, deletion mutant DHFR or its complexes with ligand(s). \square , native enzyme alone; Δ , enzyme + folate; \circ , enzyme + NADPH; \times , enzyme + MTX + NADPH. The antibody solutions were then applied directly onto the microtiter plates and assayed as described under “Experimental Procedures.” A_0 , absorbance in the absence of either wild-type or mutant DHFRs during the preincubation; A, absorbance in the presence of various forms of wild-type or mutant DHFRs at the indicated concentration.

type had much less effect on the antibody binding to the native enzyme (Fig. 6B, I–III), especially in the binary complexes. The data in Fig. 6 are described in Table II in terms of the concentration of native wild-type or mutant hDHFR needed to inhibit antibody binding to denatured hDHFR by approximately 50% either in the absence or in the presence of various ligands. The values for wild type–ligand complexes range from 2-fold to over 16-fold greater than the values for the deletion mutant.

DISCUSSION

Studies of ligand-induced conformational changes in DHFR deduced from kinetic studies (36, 37) and NMR or fluorescence experiments (5–9, 38) have focused on bacterial enzymes. Changes have been localized in the environment of amino acid residues located outside the active site upon the binding of NADP $^{+}$, NADPH, and anti-folates such as trimethoprim and MTX. In order to understand the conformational changes of hDHFR and the role of “loop” regions in such changes as well as in enzyme function, we have constructed, by site-directed mutagenesis, a hDHFR mutant, des[Gly 45 -Lys 46], with a 2-residue deletion in a loop region consisting of residues 40–46. The x-ray structure of hDHFR showed that these 2 residues are distant from the active site

of the enzyme (16). The thermal parameter main chain B value for this highly exposed loop region is 0.376 nm 2 , compared with 0.119 nm 2 for the rigid core of the protein, indicating that this region has a high thermal mobility. The conformational changes of DHFR caused by either ligand binding or activating agents were most probably occurring in mobile regions of protein. Since certain antigenic sites must be mobile for recognition by antibody, these sites might be involved in such conformational changes. Experiments using anti-peptide antibodies showed that the wild-type hDHFR, in the presence of NADPH, folate, or MTX and NADPH, bound significantly less of the antibodies than the apoenzyme (cf. Fig. 6). The antigenic sites became either inaccessible or lost sufficient flexibility for antibody binding due to the conformational changes in DHFR (14). From results of peptide mapping, Gly 45 and Lys 46 are part of an antigenic epitope (15). This information suggests that the loop region 40–46 is involved in the overall conformational constraints of the enzyme molecule.

From steady-state kinetic studies (Table I), the K_m values of the deletion mutant DHFR for both FAH $_2$ and NADPH are similar to those of the wild-type enzyme. The results suggest that deletion of 2 residues in the loop region 40–46 has little or no effect on substrate binding. This was not totally unexpected since this loop is far from the substrate binding site cleft. In contrast, the V_{max} value of des[Gly 45 -Lys 46] is 2.8-fold lower than that of the wild type. This observation may indicate that the loss of 2 residues from the loop region 40–46 has decreased the flexibility of this region which, in turn, results in a decrease in enzyme catalytic efficiency. The des[Gly 45 -Lys 46] mutant, in its apoenzyme form, is very unstable (Fig. 1) and in the absence of substrate, activity is rapidly lost. Due to this property, we were unable to determine the K_d values of mutant enzyme for substrate and cofactor. Crystal structure studies of chicken liver DHFR, which has a structure similar to hDHFR, indicate that there is a nonbonded contact between loop 40–46 and another loop region at residues 100–108 (17). This contact might be disrupted due to the deletion of Gly 45 -Lys 46 in the loop 40–46. This nonbonded contact could be an important factor for maintaining the enzyme tertiary structure.

As with most DHFRs from vertebrate sources, hDHFR can be activated over a limited concentration range by salt, urea, and organomercurial compounds such as pHMB (4, 34). Investigations on the mechanism of activation have suggested that a general unfolding of the native protein is a common feature (19, 39, 40). Compared with wild type enzyme, activation of des[Gly 45 -Lys 46] hDHFR by KCl or urea is much less at each corresponding concentration (Fig. 4, A and B). Unlike wild type, the mutant DHFR was not activated by pHMB (Fig. 5). The reduction in the ability to be activated by these agents in the case of the mutant enzyme might be due to the 2-residue deletion in loop 40–46 impairing the

TABLE II
Concentration of wild-type and des[Gly 45 -Lys 46] DHFRs and their complexes with ligands required for 50% inhibition of anti-peptide antibody binding to denatured hDHFR
Concentration is expressed in micrograms per milliliter. The values shown in this table are derived from Fig. 6.

Peptide immunogen	Ligand											
	None ^a			Folate			NADPH			MTX + NADPH		
	WT	DM	WT/DM	WT	DM	WT/DM	WT	DM	WT/DM	WT	DM	WT/DM
15–52	4.2	3.3	1.27	18.0	4.0	4.5	36	5.5	6.54	38	18	2.11
53–111	3.8	2.6	1.46	>50	3	>16	>50	3	>16	>40	4.5	>11
140–186	5.8	3.5	1.65	40	4.5	8.89	40	4.5	8.89	40	16	2.5

^a WT, wild type; DM, deletion mutant.

limited unfolding of the enzyme molecule. In other words, the loop might be involved in the unfolding process of wild-type enzyme which occurs concomitantly with activation. Additionally, deletion mutant activity decreased rapidly at higher salt and urea concentrations at which the wild-type enzyme still can be activated. The mutant is also much more susceptible to denaturation which is consistent with the instability of this enzyme.

To probe the conformational states of the mutant and wild-type enzymes and their ligand complexes we have employed competitive ELISA using sequence-specific anti-human DHFR peptide antibodies. The cyanogen bromide peptides from hDHFR were homogeneous and corresponded to the sequences 15–52, 53–111, and the COOH-terminal sequence 140–186 (14). Antisera against these peptides were highly specific for the corresponding peptides. The experiments indicate that, upon binding to the various ligands, wild-type enzyme exhibits a significant reduction in the ability to bind to the three peptide antibodies. The inhibition occurred to various extents with the different ligands used and, in most cases, ternary complexes bound less antibody than binary complexes (Fig. 6 and Table II). Ratnam *et al.* (14) have demonstrated that the ligands alone have no detectable effect on antibody binding to denatured DHFR immobilized in the microtiter plates and no direct interference with antibody binding in solution. The loss of antigenicity indicates either that the region where the antibody binds has become sterically inaccessible to the peptide antibody or that it has adopted a significantly different or more constrained conformation (41). The dramatic reduction in antibody binding suggests that conformational changes induced by ligands have indeed occurred and that these changes involve different portions of the protein molecule, since the binding of all three anti-peptide antibodies was decreased following ligand binding. In contrast, the binding of ligand(s) with the deletion mutant enzyme had much less effect on the inhibition of antibody binding, especially in binary complexes (Fig. 6). Ligand-bound des[Gly⁴⁵-Lys⁴⁶] achieved about a 90% inhibition at a 50 µg/ml concentration as did the apoenzyme, but ligand-bound wild type only achieved about 70% inhibition at the same protein concentration. To achieve 50% inhibition required at least a 2-fold greater amount of protein of ligand-bound wild type as it did for the corresponding ligand-bound mutant enzyme. These results clearly indicate that the conformational changes of wild type induced by ligand are more pronounced than that of the mutant. The results also show that the 2-residue deletion in the 40–46-loop region has affected the antibody binding not only in the peptide 15–52 region but also in the peptide 53–111 and 140–186 regions. Thus, it appears that the loop region 40–46 is important for both modulating conformational changes in the protein and for communicating the effects of ligand binding.

In general, our studies on the deletion mutant des[Gly⁴⁵-Lys⁴⁶] suggest that the flexible loop between residues 40 and 46 plays an important role in ligand-induced conformational changes in the DHFR molecule and, perhaps, in the partial unfolding of the protein during activation. The loss of flexibility in this loop region results in specific constraints on the conformational changes which can occur in the enzyme molecule upon ligand binding. The deletions also result in a decrease in the catalytic efficiency of the enzyme. Little effect on the apparent values of K_m for both FAH₂ and NADPH were observed implying that the loop region 40–46 may be more involved in catalytic efficiency than in substrate or coenzyme binding to hDHFR.

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REFERENCES

- Freisheim, J. H., and Matthews, D. A. (1984) in *Folate Antagonists as Therapeutic Agents* (Sirotnak, F. M., Burchall, J. J., Ensminger, W. D., and Montgomery, J. A., eds) Vol. 1, pp. 69–131, Academic Press, Orlando, FL
- Hitchings, G. H., and Baccanari, D. P. (1984) in *Folate Antagonists as Therapeutic Agents* (Sirotnak, F. M., Burchall, J. J., Ensminger, W. D., and Montgomery, J. A., eds) Vol. 1, pp. 151–172, Academic Press, Orlando, FL
- Montgomery, J. A., and Piper, J. R. (1984) in *Folate Antagonists as Therapeutic Agents* (Sirotnak, F. J., Burchall, J. J., Ensminger, W. D., and Montgomery, J. A., eds) Vol. 1, pp. 219–260, Academic Press, Orlando, FL
- Blakley, R. L. (1984) in *Folates and Pterins* (Blakley, R. L., and Benkovic, S. J., eds) Vol. 1, pp. 191–253, John Wiley and Sons, New York
- Bevan, A. W., Birdsall, B., Roberts, G. C. K., Feeney, J., Gronenborn, A., Clore, G. M., and Burgen, A. S. V. (1983) *Chemistry and Biology of Pteridines: Proceedings of the Seventh International Symposium on Pteridines and Folic Acid Derivatives. Chemical, Biological and Clinical Aspects.* (Blair, J. A., ed) pp. 557–561, Walter de Gruyter, Berlin
- Birdsall, B., Burgen, A. S. V., and Roberts, G. C. K. (1980) *Biochemistry* **19**, 3732–3737
- Birdsall, B., Bevan, A. W., Pascual, C., Roberts, G. C. K., Feeney, J., Gronenborn, A., and Clore, G. M. (1984) *Biochemistry* **23**, 4733–4742
- Gronenborn, A., Birdsall, B., Hyde, E. I., Roberts, G. C. K., Feeney, J., and Burgen, A. S. V. (1981) *Biochemistry* **20**, 1717–1722
- Feeney, J., Roberts, G. C. K., Thomson, J. W., King, R. W., Griffiths, D. V., and Burgen, A. S. V. (1980) *Biochemistry* **19**, 2316–2321
- Matthews, D. A., Alden, R. A., Freer, S. T., Xuong, N., and Kraut, J. (1979) *J. Biol. Chem.* **254**, 4144–4151
- Matthews, D. A., Bolin, J. T., Burridge, J. M., Felman, D. J., Volz, K. W., Kaufman, B. T., Beddel, C. R., Champness, J. N., Stammers, D. K., and Kraut, J. (1985) *J. Biol. Chem.* **260**, 381–391
- Matthews, D. A., Bolin, J. T., Burridge, J. M., Filman, D. J., Volz, K. W., and Kraut, J. (1985) *J. Biol. Chem.* **260**, 392–399
- Ratnam, M., Delcamp, T. J., and Freisheim, J. H. (1986) *Biochemistry* **25**, 5453–5459
- Ratnam, M., Tan, X., Prendergast, N. J., Smith, P. L., and Freisheim, J. H. (1988) *Biochemistry* **27**, 4800–4804
- Tan, X., Ratnam, M., Huang, S., Smith, P. L., and Freisheim, J. H. (1990) *J. Biol. Chem.* **265**, 8022–8026
- Oefner, C., D'Arcy, A., and Winkler, F. K. (1988) *Eur. J. Biochem.* **174**, 377–385
- Volz, K. W., Matthews, D. A., Alden, R. A., Freer, S. T., Hansch, C. C., Kaufman, B. T., and Kraut, J. (1982) *J. Biol. Chem.* **257**, 2528–2536
- Blakley, R. L. (1960) *Nature* **188**, 231–232
- Prendergast, N. J., Delcamp, T. J., Smith, P. L., and Freisheim, J. H. (1988) *Biochemistry* **27**, 3664–3671
- Sanger, F., Nicklen, S., and Coulson, A. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
- Biggin, M. D., Gibson, T. J., and Hong, G. J. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80**, 3963–3965
- Agellon, L., and Chen, T. (1986) *Gene Anal. Tech.* **3**, 86–89
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Williams, J. W., Morrison, J. F., and Duggleby, R. G. (1979) *Biochemistry* **18**, 2567–2573
- Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- Seeger, D. R., Cosulich, D. B., Smith, J. M., and Hultquist, M. E. (1949) *J. Am. Chem. Soc.* **71**, 1753–1758
- Kempton, R. J., Black, A. M., Amstead, G. M., Kumar, A. A., Blankenship, D. T., and Freisheim, J. H. (1982) *J. Med. Chem.* **24**, 475–477
- Appleman, J. R., Beard, W. A., Delcamp, T. J., Prendergast, N. J., Freisheim, J. H., and Blakley, R. L. (1989) *J. Biol. Chem.* **264**, 2625–2633

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29. Horecker, B. L., and Kornberg, A. (1948) *J. Biol. Chem.* **175**, 385-390
30. Hochschwender, S., Langeborg, L. K., Schneider, D. W., and Lindstrom, J. M. (1985) in *Hybridomas in Biotechnology and Medicine* (Springer, T., ed) pp. 223-238, Plenum Publishing Corp., New York
31. Nakamaye, K., and Eckstein, F. (1986) *Nucleic Acids Res.* **14**, 9679-9698
32. Sayers, J. R., Schmidt, W., and Eckstein, F. (1988) *Nucleic Acids Res.* **16**, 791-802
33. Huang, S., Delcamp, T. J., Tan, X., Smith, P. L., Prendergast, N. J., and Freisheim, J. H. (1989) *Biochemistry* **28**, 471-478
34. Delcamp, T. J., Susten, S. S., Blankenship, P. T., and Freisheim, J. H. (1983) *Biochemistry* **22**, 633-639
35. Schweitzer, B. I., Sirmat Kandada, S., Gritsman, H., Sheridan, R., Vankataraghavan, R., and Bertino, J. R. (1990) *J. Biol. Chem.* **264**, 20786-20795
36. Penner, M. H., and Freiden, C. (1985) *J. Biol. Chem.* **260**, 5366-5369
37. Dunn, S. M. J., and King, R. W. (1980) *Biochemistry* **19**, 766-773
38. Kimber, B. J., Griffiths, D. V., Birdsall, B., King, R. W., Scudder, P., Feeney, J., Roberts, G. C. K., and Burgen, A. S. V. (1977) *Biochemistry* **16**, 3492-3500
39. Huennekens, F. M., Mell, G. P., Harding, N. G. L., Gundersen, L. E., and Freisheim, J. H. (1970) in *Chemistry and Biology of Pteridines* (Iwai, K., Akino, M., Groto, M., and Iwanami, Y., eds) pp. 329-350, International Academic Printing, Tokyo
40. Barbehenn, E. K., and Kaufman, B. T. (1982) *Arch. Biochem. Biophys.* **219**, 236-247
41. Gariépy, J., Mietzner, T. A., and Schoolnik, G. K. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 8888-8892